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Left Ventricular Diastolic Dysfunction During Demand Ischemia: Rigor Underlies Increased Stiffness Without Calcium-Mediated Tension. Amelioration by Glycolytic Substrate

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OBJECTIVES The goal of this study was to determine the subcellular mechanism(s) underlying increased left ventricular (LV) diastolic chamber stiffness (DCS) during angina (demand ischemia).

BACKGROUND Increased DCS may result from increased diastolic myocyte calcium concentration and/or rigor. Therefore, we assessed the effects of direct alterations of both calcium-activated tension and high-energy phosphates on increased DCS.

METHODS Demand ischemia was reproduced in isolated, isovolumic, red-cell perfused rabbit hearts by imposing low-flow ischemia and pacing tachycardia. This resulted in increased DCS. Interventions were performed after LV end-diastolic pressure had increased approximately 7 mm Hg. Initially, to determine the effects of altered calcium concentration or myofilament calcium responsiveness, hearts received either: 1) 5 or 14 mmol/L calcium chloride; 2) 8 mmol/L egtazic acid; 3) 5 mmol/L butane-dione-monoxime (BDM); or 4) 50 mmol/L ammonium chloride (NH_4Cl). Then, to assess the contribution of decreased high-energy phosphate supply, hearts received 5) glucose (25 mmol/L) and insulin (400 μU/ml).

RESULTS 1) Calcium chloride, 5 and 14 mmol/L, increased LV systolic pressure by 42% and 70%, respectively ($p < 0.001$), indicating increased calcium-activated tension, but did not further increase DCS, implying intact diastolic calcium resequestration. 2) Egtazic acid reduced LV systolic pressure by 30% ($p < 0.001$), indicating reduced intracellular calcium, but failed to reduce increased DCS. 3) Butane-dione-monoxime and NH_4Cl chloride affected contractile function (i.e., a calcium-driven force) but did not alter increased DCS. 4) Glucose and insulin, which increase high-energy phosphates during ischemia, reduced increased DCS by 50% ($p < 0.001$).

CONCLUSIONS Increased DCS during demand ischemia was insensitive to maneuvers altering intracellular calcium concentration or myofilament calcium-responsiveness, that is, evidence against an etiology of calcium-activated tension. In contrast, increased glycolytic substrate ameliorated increased DCS, supporting a primary mechanism of rigor-bond formation. (J Am Coll Cardiol 2001;37:2144–53) © 2001 by the American College of Cardiology

Increased left ventricular (LV) diastolic chamber stiffness occurs during angina (1,2). This is accompanied by reduced lung compliance (3) with the clinical sequelae of chest constriction and, ultimately, pulmonary edema, as described by Osler (4). Cytosolic calcium overload, with diastolic persistence of calcium-activated cross-bridge cycling, and thus diastolic myofilament tension generation, has been favored to be the underlying mechanism (5,6). Alternatively, increased diastolic tension also may result when adenosine triphosphate (ATP) is reduced (7,8), possibly in conjunction with increased adenosine diphosphate (9), resulting in failure of the actino-myosin complex to dissociate during the cross-bridge cycle. Our previous report, using quick-length changes in a model reproducing the metabolic and mechanical diastolic pathophysiology characteristic of angina, supported such a mechanism of rigor-bond formation for increased diastolic stiffness (10). Here, we used a different approach to characterize these subcellular mechanism(s) further. We tested the hypothesis that increased diastolic myofilament tension was not generated by a calcium-mediated process, by directly assessing responses to deliberate alterations of both calcium concentration, with calcium and egtazic acid (EGTA), and myofilament calcium-responsiveness, with butane-dione-monoxime (BDM) and ammonium chloride (NH_4Cl). To test for a rigor-mediated mechanism due to disordered high-energy phosphate metabolism, we determined effects of increased glycolytic substrate, which increases ATP and phosphocreatine concentrations during ischemia (11).

The results provide additional evidence for a primary mechanism involving rigor-bond formation, without any component of calcium-mediated tension, as responsible for the increased diastolic chamber stiffness observed during demand ischemia, and they extend support for a therapeutic role for glycolytic substrate.

METHODS

The model, utilizing isolated, balloon-in-LV erythrocyte-perfused rabbit hearts has been described in detail previously

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Abbreviations and Acronyms

ATP = adenosine triphosphate
BDM = butane-dione-monoxime
Ca^{++} = calcium chloride
EGTA = egtazic acid
G + I = glucose and insulin
LV = left ventricle or left ventricular
LVEDP = left ventricular end-diastolic pressure
NH_4Cl = ammonium chloride
N Sal = normal saline
+dP/dt = peak positive derivative of left ventricular pressure
−dP/dt = peak negative derivative of left ventricular pressure

(10). In each heart, baseline balloon volume was set to achieve an LV end-diastolic pressure (LVEDP) of 20 mm Hg, and, thereafter, the volume was not changed during the remainder of the experiment (i.e., each heart contracted isovolumically). Thus, LV diastolic chamber stiffness was indexed by LVEDP and LV contractility by LV systolic or developed (i.e., systolic minus diastolic) pressure and by the peak positive derivative of LV pressure (+dP/dt). Ischemia was imposed by reducing coronary blood flow to a constant rate, eliciting a coronary artery perfusion pressure of 20 mm Hg, and, thereafter, flow was not changed. After 5 min of ischemia, the demand ischemia state was created by imposing tachycardia (7 Hz) during continued restricted coronary blood flow. This resulted in a progressive increase in LVEDP. Neither tachycardia alone nor coronary flow restriction alone increased LVEDP; only the combination (i.e., demand ischemia) increased LVEDP (10). When LVEDP had increased approximately 7 mm Hg above the pre-tachycardia value, tachycardia was terminated (pacing rate was returned to 3 Hz), but coronary flow continued at its constant, reduced rate for 5 min. In this post-tachycardia hypoperfusion period, increased LVEDP persisted, representing a state of stable increased diastolic chamber stiffness resulting from increased metabolic demand during low-flow perfusion. To determine the etiology of increased stiffness, interventions were imposed after tachycardia (i.e., in the presence of sustained ischemic diastolic dysfunction). Tachycardia was terminated to achieve a diastolic period of adequate length to ensure that LVEDP was measured on the flat portion of the diastolic pressure tracing (i.e., it reflected end-diastolic tension and was not increased because of an inadequate time for complete LV relaxation to occur).

Interventions. Hemodynamic function after tachycardia during continued low-flow perfusion at a constant rate was assessed for 5 min in a group of hearts receiving no intervention (n = 6). In others, interventional agents (diluted in saline) were delivered directly into the coronary circulation via aortic infusion. Agent infusion rates never exceeded 5% of coronary flow. Concentrations are expressed below as final arterial concentrations delivered.

Altered calcium availability. To test effects of deliberate alterations of calcium concentration on increased diastolic chamber stiffness, hearts received either calcium to increase or EGTA to reduce intracellular calcium concentration. Altering extracellular calcium concentration promptly alters the intracellular calcium transient and contractile force, simultaneously and proportionately (12,13). Left ventricular contractile function, indexed by systolic or developed pressure, is known to be generated by calcium-activated cross-bridge cycling. Since the hearts contracted isovolumically and preload was constant, any change in contractile function upon exposure to calcium or EGTA represented an alteration of calcium-mediated active tension. Hence, in these experiments, alterations of LV contractile function were used as an "internal control," reflecting a calcium-myofilament interaction, and were compared with simultaneous effects (if any) on increased isovolumic LVEDP to assess whether this was similarly calcium-dependent.

Calcium. Hearts received a 1 min infusion of: 1) normal saline (N Sal) (n = 7), 2) 5 mmol/L calcium chloride (Ca^{++}) (5 mM, n = 7) and 3) 14 mmol/L Ca^{++} (14 mM, n = 6). These infusions commenced immediately after cessation of tachycardia.

EGTA. Hearts received a 1 min infusion of 8 mmol/L EGTA 1.5 min after tachycardia was terminated (EGTA, n = 6). A post-tachycardia period of 1.5 min was selected because, at this point, contractile function, depressed during tachycardia, had recovered and stabilized, therefore allowing manifestation of EGTA's effect on contractile function.

Myofilament calcium-responsiveness was altered by agents that do not affect intracellular calcium concentration but manifest effects on myofilament calcium-sensitivity at both systolic and diastolic levels of calcium (14). Again, changes in isovolumic LV contractile function (dependent on calcium-activated cross-bridge cycling) in response to these interventions represented effects on contractility at the myofilament level and provided an "internal control" to compare with effects (if any) occurring on increased isovolumic LVEDP.

NH_4Cl. Since myofilament sensitivity to calcium is influenced by intracellular pH, we used NH_4Cl, which produces intracellular alkalanization, followed by washout acidification (14). We hypothesized that, if increased diastolic stiffness was calcium-driven, then NH_4Cl should exert a biphasic effect on isovolumic LVEDP. Hearts received a 50 mmol/L infusion for 1 min commenced 1.5 min after tachycardia (NH_4Cl, n = 7).

BDM. Butane-dione-monoxime reduces calcium-activated cross-bridge cycling without altering the calcium transient (at concentrations ≤5 mmol/L) (i.e., results in excitation-contraction uncoupling and is incapable of breaking formed rigor-bonds) (14,15). We reasoned that, if increased diastolic stiffness due to demand ischemia resulted from persistent calcium-activated tension, then this should be reduced by BDM. To test this, hearts received a 5 mmol/L infusion for 5 min after tachycardia (BDM, n = 8).

Table 1. Preintervention Characteristics of Hearts Subjected to Demand Ischemia

Groups	Size	CBF ml/min/g LVww		Tachycardia Duration (min)	Net Post-tachycardia Increase in LVEDP (mm Hg)
		Baseline	Ischemia		
N Sal	(n = 7)	1.05 ± 0.08	0.34 ± 0.04	14.6 ± 1.8	7.0 ± 0.4
Ca^{++} 5 mM	(n = 7)	1.06 ± 0.04	0.31 ± 0.03	14.3 ± 1.7	7.6 ± 0.9
Ca^{++} 14 mM	(n = 6)	1.06 ± 0.12	0.33 ± 0.03	15.8 ± 3.8	8.4 ± 1.3
EGTA	(n = 6)	0.98 ± 0.06	0.32 ± 0.02	16.1 ± 1.4	6.4 ± 0.4
NH_4CL	(n = 7)	1.22 ± 0.11	0.40 ± 0.05	15.7 ± 2.2	8.9 ± 1.3
BDM	(n = 8)	1.05 ± 0.03	0.34 ± 0.01	15.3 ± 0.9	7.3 ± 0.3
G + I	(n = 6)	0.96 ± 0.18	0.40 ± 0.02	17.3 ± 1.3	7.9 ± 0.9
Mannitol	(n = 6)	1.00 ± 0.07	0.37 ± 0.04	13.0 ± 1.6	7.7 ± 0.6

BDM = hearts receiving 5 mmol/L butane-dione-monoxime; Ca^{++} 5 mM = hearts receiving 5 mmol/L calcium; Ca^{++} 14 mM = hearts receiving 14 mmol/L calcium; CBF = coronary blood flow; EGTA = hearts receiving 8 mmol/L egtazic acid; G + I = hearts receiving high glucose (25 mmol/L) and insulin (400 μU/ml); LVEDP = left ventricular diastolic pressure; LVww = left ventricular wet weight; Mannitol = hearts receiving 25 mmol/L mannitol; NH_4CL = hearts receiving 50 mmol/L ammonium chloride; N Sal = hearts receiving saline.

Increased glycolytic substrate. To test whether disordered high-energy phosphate metabolism was responsible for increased diastolic stiffness, hearts received high glucose (25 mmol/L) and insulin (400 μU/ml) (G + I) for 5 min after tachycardia (n = 6). To control for the high osmolality of this glycolytic substrate, a group of hearts received 25 mmol/L mannitol (n = 6) for 5 min after tachycardia.

Data acquisition and statistical analysis. Pressure measurements were recorded continuously. In individual hearts, data are reported at six time points: baseline, 5 min after imposition of ischemia and 0, 1.5, 3 and 5 min after termination of tachycardia (mean ± SEM). Statistical comparisons between groups were performed by two-way analysis of variance. If overall analysis of variance indicated a significant difference of groups, trials or interaction, values at specific time points were examined by the method of least significant differences. A p value of <0.05 was considered significant.

All animal handling and procedures strictly complied with the regulations of Boston University Animal Care and the National Society for Medical Research.

RESULTS

Coronary flows and hemodynamics at baseline and before intervention were similar in all groups (Tables 1 and 2). In each heart, coronary flow and perfusion pressure remained constant during ischemia and were unaffected by tachycardia or by interventions. Hence, in our experiments, altered isovolumic LVEDP in response to interventions indicated an effect on diastolic stiffness without any confounding contribution from altered coronary vascular turgor. Post-tachycardia hemodynamic function did not differ between hearts that received no intervention compared with those that received N Sal (i.e., saline had no effect). In these, contractile function recovered and stabilized within 90 s of termination of tachycardia. Increased diastolic stiffness persisted and did not increase further (isovolumic LVEDP: N Sal, 0 vs. 5 min after tachycardia = 25 ± 1 vs. 27 ± 1 mm Hg, p = NS). This represented a state of stable ischemic diastolic dysfunction during which interventions were performed. Effects of interventions that used a saline vehicle are compared with N Sal in Table 2.

Calcium. Increasing perfusate calcium level increased contractile function, indicating a significant increase in intracellular calcium; LV systolic pressure increased to 81 ± 4 mm Hg in Ca^{++} 5 mM and to 97 ± 5 mm Hg in Ca^{++} 14 mM groups compared with 57 ± 3 mm Hg in the N Sal group ($p < 0.001$) (Fig. 1). However, LVEDP was unaffected by 5 mM Ca^{++} and significantly decreased by 14 mM Ca^{++}. The rate of LV pressure decay (−dP/dt), which had decreased with the onset of ischemia, further decreased with tachycardia. However, both 5 mmol/L and 14 mmol/L of calcium significantly enhanced −dP/dt.

EGTA reduced contractile function; LV systolic pressure decreased by 30% to 42 ± 5 mm Hg compared with 60 ± 3 mm Hg in the N Sal group ($p < 0.001$), indicating decreased intracellular calcium concentration (Fig. 2). However, there was no reduction of increased LVEDP.

Ammonium chloride elicited a biphasic response of contractile function: an initially positive, followed by a negative, inotropic effect (Fig. 3). The peak positive inotropic effect occurred 30 s after infusion, consistent with an initial alkalanization, where systolic pressure increased from 55 ± 3 to 70 ± 4 ($p < 0.001$), indicating enhanced myofilament calcium-sensitivity, and was accompanied by increased +dP/dt. However, there was no concomitant change in increased LVEDP. A negative inotropic effect then followed (during "washout" acidification), where systolic pressure decreased from 70 ± 4 to 52 ± 2 mm Hg ($p < 0.001$), indicating diminished myofilament calcium-sensitivity but, again, with no accompanying change in increased LVEDP.

Butane-dione-monoxime progressively reduced contractile function, consistent with its property of reducing calcium-driven cross-bridge-cycling (Fig. 4). Left ventricular systolic pressure decreased from 60 ± 2 to 55 ± 2 ($p < 0.01$ vs. N Sal). Developed pressure decreased by approximately 25%, from 31 ± 3.4 to 23 ± 2.6 mm Hg ($p < 0.001$) at 5 min. However, increased LVEDP was not similarly reduced (i.e., increased diastolic stiffness was not driven by a calcium-mediated process).

Hence, interventions affecting calcium concentrations

Table 2. Hemodynamic Effects of Interventions

Group	Baseline	Ischemia	Post-tachycardia (min)			
			0	1.5	3	5
LVSP (mm Hg)						
N Sal	126 ± 4	67 ± 5	38 ± 1	57 ± 3	60 ± 3	61 ± 4
Ca^{++} 5 mM	130 ± 6	61 ± 3	39 ± 1	81 ± 4*	69 ± 3†	68 ± 2‖
Ca^{++} 14 mM	119 ± 6	67 ± 2	43 ± 2	97 ± 5*	70 ± 6*	68 ± 4
EGTA	122 ± 3	62 ± 5	39 ± 2	58 ± 3	42 ± 5*	51 ± 5‡
NH_4CL	123 ± 3	58 ± 3	39 ± 1	55 ± 3	70 ± 4*	52 ± 2*
BDM	120 ± 6	63 ± 3	42 ± 2	60 ± 2	59 ± 2	55 ± 2‡
G + I	122 ± 10	65 ± 4	46 ± 4	63 ± 4	69 ± 4	69 ± 4
Mannitol	117 ± 4	57 ± 2	37 ± 3	53 ± 3	57 ± 4	59 ± 4
LVEDP (mm Hg)						
N Sal	20	18 ± 0.4	25 ± 1	25 ± 1	26 ± 1	27 ± 1
Ca^{++} 5 mM	20	16 ± 0.5	23 ± 1	22 ± 1	23 ± 2	24 ± 2
Ca^{++} 14 mM	20	17 ± 1	24 ± 1	21 ± 1*	21 ± 1*	23 ± 1†
EGTA	20	16 ± 1	23 ± 1	24 ± 2	26 ± 2	30 ± 3‖
NH_4CL	20	17 ± 0.3	26 ± 1	25 ± 2	24 ± 2	26 ± 3
BDM	20	19 ± 0.4	27 ± 1	29 ± 1	30 ± 2	33 ± 3
G + I	20	17 ± 1	25 ± 1	24 ± 1	23 ± 1*	22 ± 1*
Mannitol	20	18 ± 0.4	25 ± 0.3	26 ± 1	26 ± 1	28 ± 1
LV + dP/dt (mm Hg/s)						
N Sal	1,286 ± 69	546 ± 58	182 ± 8	332 ± 25	382 ± 29	395 ± 36
Ca^{++} 5 mM	1,534 ± 108	587 ± 39	251 ± 31	787 ± 66*	597 ± 59*	566 ± 57†
Ca^{++} 14 mM	1,320 ± 144	599 ± 47	281 ± 32	1,283 ± 98*	724 ± 114*	627 ± 80‡
EGTA	1,372 ± 58	605 ± 70	269 ± 48	413 ± 65	204 ± 60‡	287 ± 86
NH_4CL	1,286 ± 87	491 ± 30	202 ± 22	355 ± 45	560 ± 66†	305 ± 36
BDM	1,267 ± 110	573 ± 36	236 ± 22	390 ± 38	370 ± 30	293 ± 32§
G + I	1,390 ± 115	608 ± 34	267 ± 13	457 ± 46‡	567 ± 43*	616 ± 51*
Mannitol	1,225 ± 86	556 ± 40	223 ± 25	391 ± 59	445 ± 55	451 ± 59
LV − dP/dt (mm Hg/s)						
N Sal	1,037 ± 55	489 ± 65	126 ± 18	301 ± 32	363 ± 35	375 ± 52
Ca^{++} 5 mM	1,062 ± 56	545 ± 21	144 ± 21	604 ± 62*	442 ± 31	449 ± 34
Ca^{++} 14 mM	1,014 ± 86	599 ± 58	192 ± 20	949 ± 77*	524 ± 76‖	488 ± 79
EGTA	956 ± 33	563 ± 75	187 ± 45	329 ± 44	133 ± 42*	199 ± 58†
NH_4CL	1,011 ± 71	446 ± 38	111 ± 17	294 ± 42	425 ± 54	240 ± 31§
BDM	1,089 ± 56	551 ± 55	167 ± 18	342 ± 42	321 ± 37	264 ± 31‖
G + I	1,020 ± 78	563 ± 59	147 ± 25	356 ± 31	455 ± 29	507 ± 39§
Mannitol	937 ± 62	475 ± 38	81 ± 25	256 ± 57	299 ± 61	319 ± 65

*$p < 0.001$; †$p < 0.005$; ‡$p < 0.01$; §$p < 0.02$; ‖$p < 0.05$ vs. N Sal.

BDM = hearts receiving 5 mmol/L butane-dione-monoxime; Ca^{++} 5 mM = hearts receiving 5 mmol/L calcium; Ca^{++} 14 mM = hearts receiving 14 mmol/L calcium; EGTA = hearts receiving 8 mmol/L egtazic acid; G + I = hearts receiving high glucose (25 mmol/L) and insulin (400 μU/ml); LV = left ventricular; LV + dP/dt = peak positive derivative of LV pressure; LV − dP/dt = peak negative derivative of LV pressure; LVEDP = LV diastolic pressure; LVSP = LV systolic pressure; Mannitol = hearts receiving 25 mmol/L mannitol; NH_4CL = hearts receiving 50 mmol/L ammonium chloride; N Sal = hearts receiving saline.

directly, or its effects further "downstream" at the myofilament level, failed to alter increased diastolic stiffness resulting from demand ischemia in the direction expected of a calcium-dependent tension (in contrast with their effects on contractility).

Increased glycolytic substrate. Glucose and insulin partially reversed the ischemia-induced increase in diastolic chamber stiffness; LVEDP declined from 25 ± 1 to 22 ± 1 mm Hg 5 min after infusion ($p < 0.001$) (i.e., toward pre-tachycardia values [Fig. 5 and 6]). At 5 min after infusion, LVEDP was 27 ± 1 mm Hg in N Sal compared with 22 ± 1 mm Hg in G + I ($p < 0.001$). Since LVEDP was approximately 18 mm Hg before tachycardia in both groups, the effect of G + I represented an amelioration of ischemic diastolic dysfunction of >50% during this period. Glucose and insulin also enhanced relaxation rate; −dP/dt increased to 507 ± 39 mm Hg/s at 5 min ($p < 0.02$). Glucose and insulin did not significantly affect LV systolic pressure. However, since G + I progressively reduced LVEDP, there was an accompanying increase in LV developed pressure. This increased by 38% at 5 min (47 ± 4 vs. 34 ± 4 mm Hg in N Sal, $p < 0.01$), and +dP/dt increased to 616 ± 51 ($p < 0.001$) (i.e., a significant increase in contractile function). The effects of G + I could not be explained by its high osmolality since equiosmolar mannitol affected neither systolic nor diastolic function (p = NS vs. N Sal). Figure 6 compares the positive inotropic and lusitropic effects of G + I to equiosmotic mannitol.

DISCUSSION

Here, in a demand ischemia model simulating features of angina, we demonstrated that increased diastolic tension

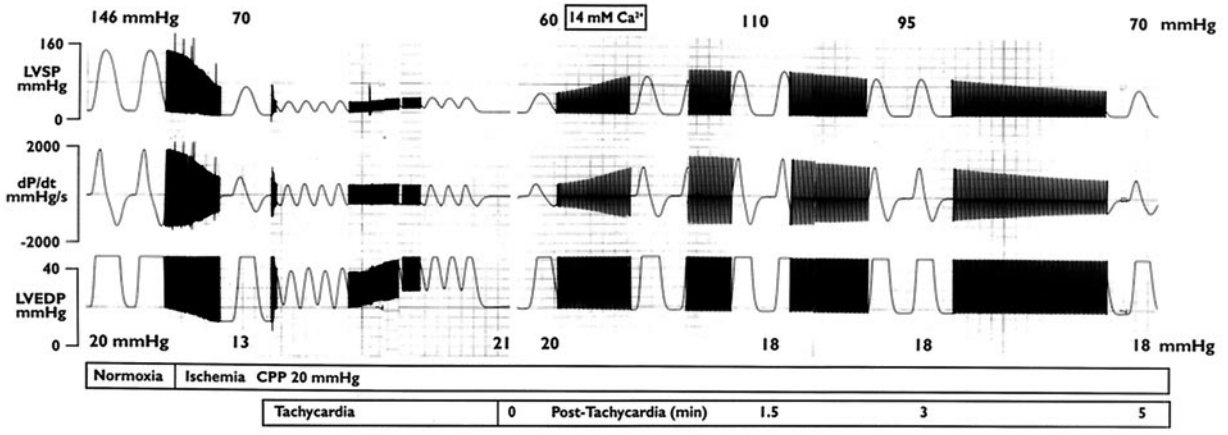

Figure 1. Calcium during demand ischemia. Ischemia (decreasing coronary perfusion pressure [CPP] to 20 mm Hg) reduced left ventricular systolic pressure (LVSP) to 70 mm Hg and left ventricular end-diastolic pressure (LVEDP) to 13 mm Hg. Tachycardia increased isovolumic LVEDP to 20 mm Hg, indicating increased diastolic chamber stiffness. A 14 mmol/L calcium (14 mM Ca^{++}) imposed after tachycardia during sustained ischemic diastolic dysfunction increased LVSP from 60 to 110 mm Hg, indicating increased intracellular calcium concentration. However, LVEDP did not simultaneously increase (but in this example decreased from 20 to 18 mm Hg), suggesting that during ischemic diastolic dysfunction, calcium resequestration mechanisms remained intact and that the upward shift in LVEDP during tachycardia was not calcium-driven. Left ventricular systolic pressure recovered subsequent to infusion.

was insensitive to alterations of either calcium concentration or myofilament calcium-responsiveness but was responsive to increased glycolytic substrate. This suggests that disordered high-energy phosphate metabolism, without any component of a calcium-mediated tension, is the mechanism responsible for abnormal diastolic relaxation.

Angina/demand ischemia. The characteristic reduction in diastolic distensibility during angina has been attributed to persistent diastolic calcium (1,5,6), although this has not been directly demonstrated. The demand ischemia that characterizes angina may especially predispose to cytosolic calcium overload since, not only can energy-dependent calcium clearance mechanisms (resequestration by the sarcoplasmic reticulum and transsarcolemmal efflux) be limited by ischemia, but tachycardia results in repetitive depolarizations with increased sodium and calcium entry per unit time. Tachycardia simultaneously abbreviates the diastolic period during which calcium reuptake and efflux occur.

Our experimental demand ischemia model replicated the features of a patient with fixed coronary artery stenosis who develops angina with increased myocardial oxygen demand during tachycardia. Diastolic chamber stiffness increased only when tachycardia was superimposed upon a state of fixed moderate coronary flow reduction (but not with tachycardia or ischemia alone) and was reversible when the supply-demand mismatch was corrected (10). However, in this model, the use of global (not regional) ischemia, though imposing homogenous perfusion conditions throughout the

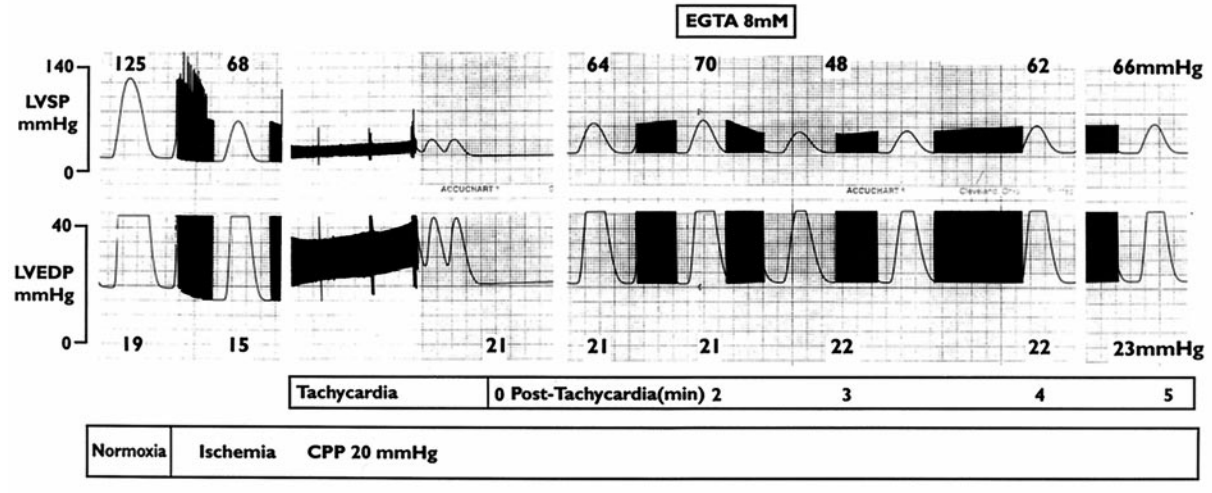

Figure 2. Egtazic acid (EGTA) during demand ischemia. Ischemia (decreasing coronary perfusion pressure [CPP] to 20 mm Hg) reduced left ventricular systolic pressure (LVSP) to 68 mm Hg and left ventricular end-diastolic pressure (LVEDP) to 15 mm Hg. Tachycardia increased LVEDP to 21 mm Hg, indicating increased diastolic chamber stiffness. An 8 mmol/L EGTA (8 mM) imposed after tachycardia during sustained ischemic diastolic dysfunction reduced LVSP from 70 to 48 mm Hg, indicating reduced intracellular calcium concentration but did not decrease elevated LVEDP, suggesting that this was not calcium-driven. Contractile function recovered after termination of EGTA.

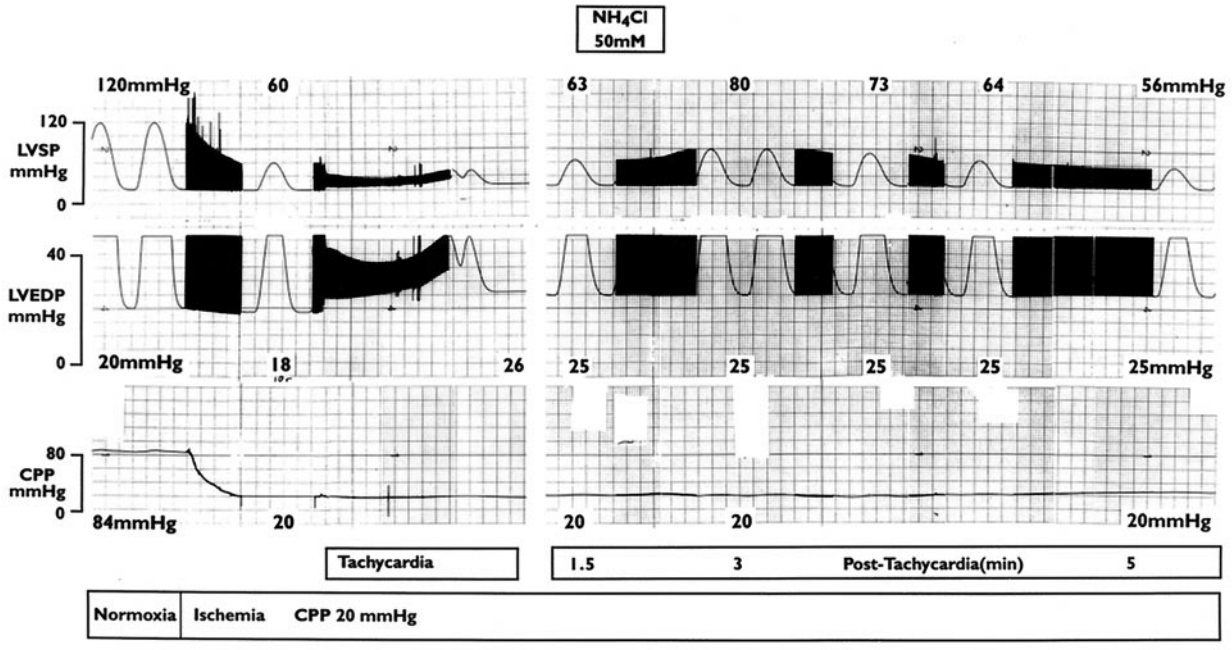

Figure 3. Ammonium chloride (NH_4Cl) during demand ischemia. Ischemia (decreasing coronary perfusion pressure [CPP] to 20 mm Hg) reduced left ventricular systolic pressure (LVSP) to 60 mm Hg and left ventricular end-diastolic pressure (LVEDP) to 18 mm Hg. Tachycardia increased LVEDP to 25 mm Hg, indicating increased diastolic chamber stiffness. 50 mmol/L ammonium chloride (50 mM NH_4Cl) imposed after tachycardia during sustained ischemic diastolic dysfunction initially increased LVSP from 63 to 80 mm Hg, then reduced it to 56 mm Hg, indicating a positive, followed by a negative, inotropic effect, consistent with its known effects on intracellular pH and, hence, myofilament calcium-sensitivity. Elevated LVEDP, however, remained unchanged, implying that it was not increased by a calcium-activated mechanism.

heart and facilitating mechanistic determination, resulted in a degree of contractile dysfunction not observed clinically unless there is accompanying three-vessel disease.

Lack of a role for calcium. Despite the hypothetical predilection for calcium overload during demand ischemia, our results demonstrated no evidence for such a state occurring in this model. Neither deliberate alterations of calcium availability (increasing perfusate calcium concentration or adding EGTA to the perfusate) nor alteration of myofilament calcium-responsiveness (with BDM or NH_4Cl) had any effect on increased diastolic stiffness in the direction expected of a calcium-mediated tension. In striking contrast, all of these interventions markedly affected LV contractile function, demonstrating that these interventions achieved their intended intracellular action on calcium-activated cross-bridge cycling and, hence, calcium-activated tension. Thus, the calcium dependence and sensitivity of systolic active tension was illustrated but clearly dissociated

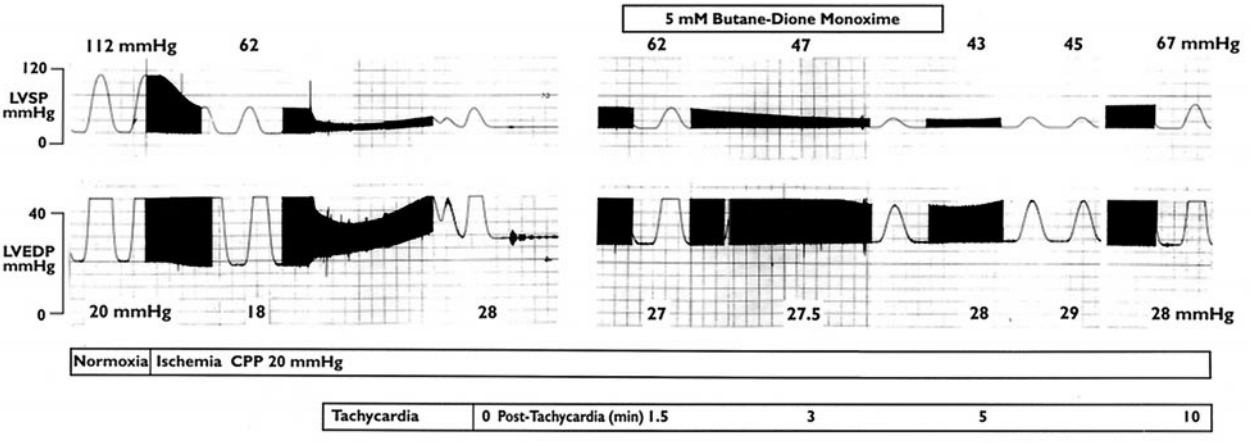

Figure 4. Butane-dione-monoxime (BDM) during demand ischemia. Ischemia (decreasing coronary perfusion pressure [CPP] to 20 mm Hg) reduced left ventricular systolic pressure (LVSP) to 62 mm Hg and left ventricular end-diastolic pressure (LVEDP) to 18 mm Hg. Tachycardia increased LVEDP to 28 mm Hg, indicating increased diastolic chamber stiffness. A 5 mmol/L BDM (5 mM) infusion imposed after tachycardia during sustained ischemic diastolic dysfunction progressively decreased LVSP from 62 to 43 mm Hg, indicating inhibition of calcium-activated myofilament cross-bridge cycling. However, elevated LVEDP was not simultaneously decreased, suggesting that it was not calcium-driven. Contractile function recovered after termination of infusion.

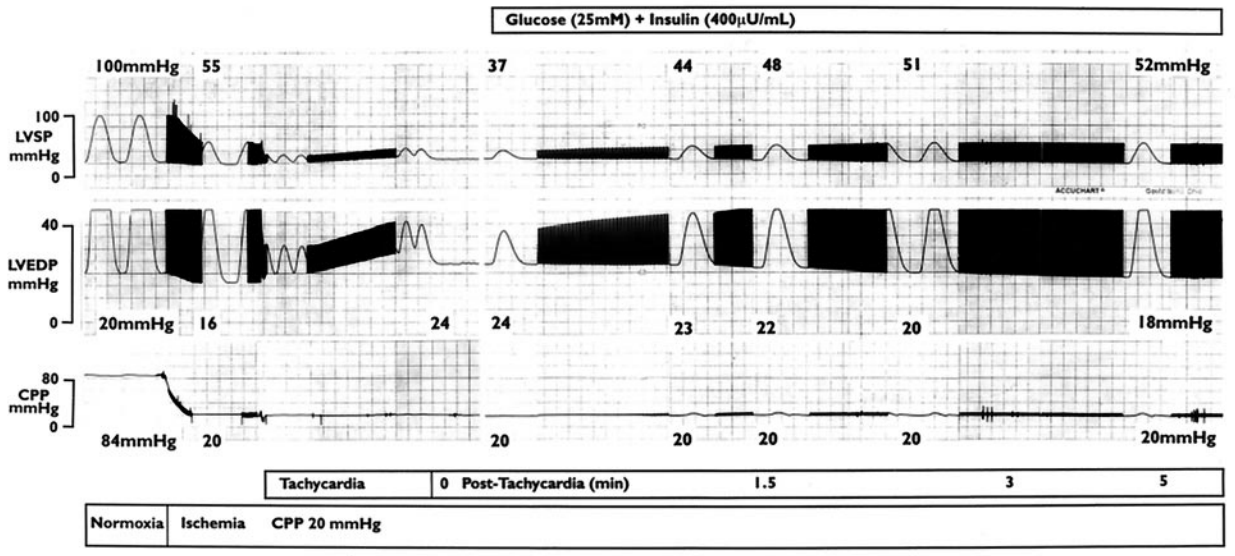

Figure 5. Increased glycolytic substrate during demand ischemia. Ischemia (decreasing coronary perfusion pressure [CPP] to 20 mm Hg) reduced left ventricular systolic pressure (LVSP) to 55 mm Hg and left ventricular end-diastolic pressure (LVEDP) to 16 mm Hg. Tachycardia increased LVEDP to 24 mm Hg, indicating increased diastolic chamber stiffness. High 25 mmol/L glucose (25 mM) and insulin (400 μU/ml) infusion gradually increased LVSP, and concomitantly decreased LVEDP from 24 to 18 mm Hg (i.e., reversed the increase in LV diastolic chamber stiffness produced by demand ischemia), toward pre-tachycardia values.

from the calcium independence and insensitivity of the persistently elevated diastolic tension. Hence, increased diastolic calcium did not appear to be a direct determinant of increased diastolic chamber stiffness. Furthermore, since deliberate calcium loading failed to drive a further increase in increased diastolic stiffness, we inferred that myocyte calcium resequestration/efflux mechanisms were able to accommodate this exogenously imposed load (i.e., were not functionally limited during this ischemic state).

The brevity of the test infusions of calcium (and EGTA) during ischemic diastolic dysfunction was a deliberate experimental design feature. Prolonged infusions may have confounded our conclusions. For example, we demonstrated preserved calcium resequestration capacity and lack of a direct relation of calcium-activated tension to increased stiffness by assessing the immediate, qualitative effects of brief alterations of calcium concentrations. Importantly, calcium loading was not used to test absolute calcium resequestration capacity. A more concentrated or prolonged calcium challenge may have ultimately driven an increase in diastolic stiffness (i.e., a response to an overwhelming exogenous calcium load), saturating diastolic clearance mechanisms. Notably, we did not reach this state even with 14 mmol/L calcium, a load sufficient to more than double contractile function. Additionally, a sustained increase in contractile work with prolonged calcium exposure during continued ischemia may have secondarily reduced ATP level, confounding interpretation of underlying mechanisms. Conversely, prolonged EGTA exposure may have increased ATP due to reduced contractile work. For example, if a sustained calcium infusion had increased LVEDP after a prolonged period of increased contractility, we would have been unable to distinguish a mechanism of a direct effect of calcium (i.e., calcium-activated tension) from increased rigor-bond formation, secondary to ATP depletion, as the mechanism underlying increased diastolic stiffness. Hence, the brevity of interventions was critical and served to assess the contribution of calcium-mediated tension to diastolic stiffness at the moment of intervention, while minimizing the possibility of secondary perturbation of high-energy phosphate metabolism.

Prior studies have demonstrated that increased diastolic calcium occurs during ischemia, hypoxia or metabolic inhibition (16,17), attributed to a combination of increased calcium influx, reduced efflux and diminished sarcoplasmic reticulum uptake. Although this seems to support a calcium-driven mechanism for ischemic diastolic dysfunction, others have reported a dissociation between the relative time course of increased myocyte calcium and the development of contracture (18). In one study, increased diastolic force preceded a rise in intracellular calcium (19) (and correlated better with reduced ATP). Thus, there may be no cause-effect relationship between the observation during ischemia of increased diastolic calcium concentration and increased diastolic tension. Our prior report suggests that diastolic calcium assessed by calcium indicators may not, in any case, accurately reflect myofilament-bound calcium (13). In contrast with previous studies, here we created the specific condition of demand ischemia and then assessed responses to interventions during established diastolic dysfunction to test the postulated relation of calcium to increased diastolic tension. Our results, however, supported no causative role for diastolic calcium-driven cross-bridge cycling as the responsible mechanism.

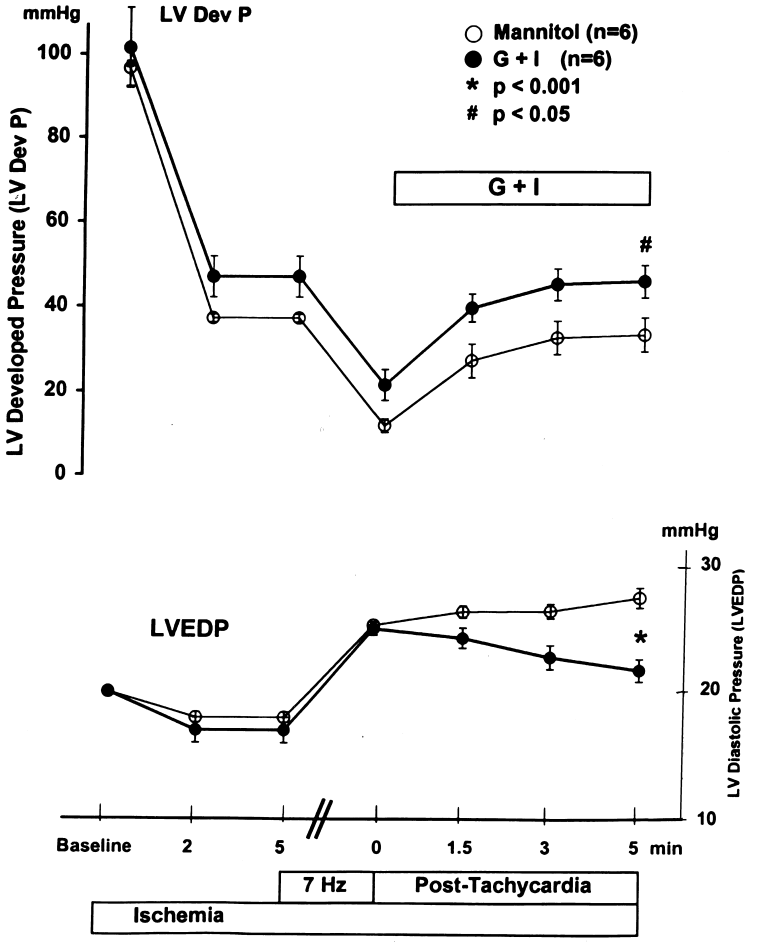

Figure 6. Summary of hemodynamic effects of increased glycolytic substrate during demand ischemia, contrasted with equiosmotic mannitol. Groups were similar before intervention. Tachycardia (7 Hz) imposed during ischemia increased diastolic stiffness (isovolumic left ventricular end-diastolic pressure [LVEDP]). Glucose 25 mmol/L and insulin 400 μU/ml (G + I) progressively reduced increased diastolic stiffness and enhanced contractile function (left ventricular developed pressure [LV Dev P]). Mannitol had no effect.

The mechanisms underlying diastolic dysfunction during the specific pathophysiology of demand ischemia have received comparatively little study. One report, in a pacing-tachycardia canine model with a fixed coronary artery stenosis, observed that caffeine administered during the last 30 s of tachycardia exacerbated the extent of diastolic dysfunction (6). In the present study, we instead used deliberate calcium loading, which is a purer challenge than caffeine (which produces sarcoplasmic reticulum dysfunction), after LVEDP had increased during demand ischemia. In contrast with results with caffeine, we could not drive a further increase in increased diastolic stiffness with this maneuver, suggesting that calcium resequestration ability had remained intact during demand ischemia, despite the increase in LVEDP.

Rigor-bond formation. Glucose and insulin ameliorated diastolic dysfunction resulting from demand ischemia. Possibly, a more prolonged exposure to glycolytic substrate (i.e., longer than the 5 min used in this study) would have totally reversed the increased chamber stiffness sustained during demand ischemia. Increasing glycolytic substrate during ischemia markedly enhances glycolytically derived ATP, increases ATP and phosphocreatine concentration and ameliorates diastolic dysfunction (11). Hypothetically, increased ATP may serve to increase calcium resequestration by ion pumps (e.g., sarcoplasmic reticular calcium-ATPase) limited by reduced energy supply during ischemia and/or act directly at the myofilament level. Since we demonstrated no evidence for disabled myocardial calcium resequestration during ischemic diastolic dysfunction, we infer that increased ATP, generated by increased glycolytic flux, enhanced relaxation directly by facilitating actino-myosin cross-bridge detachment (i.e., reversal of the actino-myosin "rigor complex" that forms in the cross-bridge cycle). Such a mechanism is consistent with our prior results where quick-length changes during demand ischemia elicited responses characteristic of rigor (10).

Myocyte heterogeneity. The development of locked cross-bridges in a minority of myocytes is a possible explanation for our results. During ischemic diastolic dysfunction, two populations of myocytes may coexist. Those in rigor, inexcitable and incapable of developing contractile force (15), increase diastolic pressure in proportion to their number but remain unresponsive to the alteration of calcium availability

or sensitivity. Myocytes not in rigor contract actively and generate phasic LV pressures but remain responsive to perturbations of calcium availability and sensitivity, manifest as alterations in contractile function. Since 5 mmol/L calcium, EGTA, BDM or NH_4Cl failed to affect elevated LVEDP after tachycardia, these ischemic, but contracting, myocytes do not seem to contribute a calcium-activated component to diastolic tension. Reversal of contracture, observed when, for example, anoxic myocytes are reoxygenated (18), manifests in the whole heart when the supply-demand mismatch is corrected or in response to glycolytic substrate, or quick-length changes (10).

Although we did not directly demonstrate intermyocyte heterogeneity in these experiments, such a concept is supported by several studies. During demand ischemia, the subendocardium is especially vulnerable to ischemia (20). Similarly, ^{31}P NMR studies of isolated hearts subjected to global low-flow ischemia identified two regions with differing degrees of acidosis, and the extent of increase in LVEDP correlated closely with the size of the more severely acidotic (presumably subendocardial) region (21). Isolated cardiomyocytes subjected to metabolic inhibition have demonstrated a variable time onset to rigor (22), and ultrastructural studies of isolated hearts subjected to global low-flow ischemia have illustrated some myocytes in rigor juxtaposed to cells with near-normal ultrastructure (23).

High-energy phosphates. Ischemic contracture in hearts subjected to zero-flow ischemia coincides with reduced ATP (7). However, in the current model, when LVEDP had increased 10 mm Hg during demand ischemia, we demonstrated no reduction in ATP beyond that attributable to the low-flow ischemic state alone (10). However, demand ischemia may represent a less extreme condition and not analogous to experimental states of either zero-flow ischemia or prolonged metabolic inhibition (in which irreversible contracture and cell death ensues relatively rapidly in all myocytes). In our model, coronary flow was only moderately reduced and myocyte function preserved manifest by continued systolic function, albeit at a reduced level ("perfusion-contraction matching"). A reversible increase in LVEDP, observed only after metabolic demand was increased during reduced perfusion, required for elicitation a critical combination of reduced coronary perfusion pressure and flow, achieved only with a red-cell perfusate at 37°C. This condition may represent a subtle ischemic state where only an increased workload reveals myocytes more vulnerable to developing rigor, the number of which progressively increases during continued supply-demand mismatch. Under such conditions, estimations of total tissue ATP may not reveal a critical ATP reduction in only the minority of myocytes that have developed locked cross-bridges. Additionally, during ischemia, ATP content does not reflect ATP turnover rate, which may better index the degree of energetic failure. Conversely, improvement of mechanical function during ischemia with increased glycolytic substrate, though associated with markedly enhanced glycolytic ATP flux, does not dramatically alter total ATP content (11).

Ramifications of the study. The study demonstrates further characteristics of actino-myosin interactions. Although rigor is typically illustrated by irreversible contracture occurring, for example, during prolonged zero-flow ischemia when there is severe ATP depletion ("stone heart") (7), our results suggest that, under selected conditions, the initial phases of the rigor process may be potentially reversible. Normally, an actino-myosin "rigor-complex" forms during the cross-bridge cycle, and detachment requires ATP binding to the high affinity site on the myosin head and is critical for achieving diastolic relaxation. During demand ischemia, when high-energy phosphate supply may be limiting despite continued myocardial perfusion, some myocytes may arrest in the rigor-phase of the cross-bridge cycle but can resume normal function when ATP supply is increased by correction of the supply-demand mismatch (10) or by provision of glycolytic substrate. Glycolytically derived ATP, therefore, facilitates myofilament relaxation independently of effects on cytosolic calcium homeostasis. This may be the mechanism underlying the beneficial effect of high glucose and insulin observed clinically in acute coronary syndromes (24) and experimentally where it blunted the degree of ischemic diastolic dysfunction sustained during prolonged underperfusion, simulating conditions of cardiogenic shock (11). In this study, we also demonstrated the ability to reverse rigor after it had occurred (with a concomitant inotropic action). Demand ischemia, classically observed during exercise-induced angina, may also occur during unstable angina or during myocardial infarction, when regions of myocardium are subjected to the combination of moderately reduced perfusion and increased metabolic demand. Therefore, our result further supports the use of glycolytic substrate as adjunctive therapy for acute coronary syndromes (24).

Study limitations. We did not measure intracellular pH, calcium or calcium-sensitivity in response to agents administered in the perfusate. However, exposure to calcium, NH_4Cl and BDM in the concentrations we used translates into the intended intracellular actions at the myofilament level, and these have been well characterized by others (12,14,15). Additionally, since only moderately reduced perfusion was imposed on hearts thus preserving active contraction, we inferred intracellular actions from changes in LV contractile pressure (i.e., a known calcium-dependent force) as an "internal control," to compare with diastolic pressure. We did not measure the effect of glycolytic substrate on high-energy phosphates, but this has been characterized previously in isolated hearts subjected to reduced perfusion conditions (11).

Summary. Our results are inconsistent with a mechanism of calcium-driven tension as the cause of the increased diastolic tension during demand ischemia, but they support a mechanism of rigor-bond formation that is ameliorated by glycolytic substrate.

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